# EXTRACTION AND CHARACTERIZATION OF ORGANIC MATTER OF A PODZOL B HORIZON *

by

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The soil studied was collected beneath a mature deciduous forest in which American beech (*Fagus grandifolia*) is a major component. The sample was obtained from the $B_{21}$ horizon of a loamy sand podzol of the St. Amable series, a soil developed from sandy material deposited in the lowland region of the province of Quebec, Canada, during the Champlain Sea episode of the late Pleistocene (7). At the site sampled the $B_{21}$ horizon was moderately well drained, uniformly reddish in colour and had an organic matter content of 5.5 per cent and pH 5.3.

For extraction, a mixture of 100 gm. air-dry soil and 300 ml. water was adjusted to pH 6.5 by addition of NaOH, 500 ml. 0.1N 8-quinolinol in benzene was added, and the system stirred mechanically for 24 hours. The pH of the aqueous phase was re-adjusted to 6.5 by addition of NaOH as necessary; at no time during the extraction did the pH fall below 6.0. After 24 hours the liquid phases were separated and the soil re-extracted in the same manner eleven times more. This treatment removed 75 to 80 per cent of the organic matter originally present; the benzene phase contained twice as much organic matter as the aqueous phase.

Each benzene phase separated was washed several times with water to remove soil particles. Each aqueous phase plus washings from the benzene phase was filtered with suction and the filtrate washed several times with $CHCl_3$ to remove traces of 8-quinolinol.

The organic matter in the combined aqueous phases obtained in extraction was fractionated using laurylpyridinium chloride (LPC) as precipitant, added as a 2.5 per cent aqueous solution at a w/w ratio of salt to organic matter of about 3 : 1. Four organic fractions were obtained viz.:

A1 — Not precipitated by LPC;

A2 — Precipitated by LPC, dissolved by 5 per cent NaCl solution, and re-precipitated by addition of 3 volumes ethanol;

A3 — Organic matter remaining in solution on addition of alcohol to precipitate the A2 fraction; and

A4 — Precipitated by LPC but not soluble in 5 per cent NaCl solution. The precipitant (LPC) was removed from these fractions by extraction with $CHCl_3$.

Removal of organic matter from the benzene phases was accomplished by, first, addition of 10 gm. Whatman standard grade cellulose powder per liter, shaking for 24 hours, filtering through sintered glass and washing the material on the filter with benzene to remove quinolinol and quinolinolates. The residue on the filter then was suspended in water and shaken for 24 hours with a 0.1N solution of 8-quinolinol in $CHCl_3$, followed by separation of the phases, filtration of the aqueous suspension on sintered glass and washing with water until the washings were colourless. This treatment transferred a large part of the organic matter from the benzene to aqueous solution. This fraction was termed B1. The organic matter remaining associated with the cellulose was removed by elution with aqueous 0.1N $NaHCO_3$ and at once neutralized to pH 6.5 to 7 by addition of acetic acid. This fraction was labelled B2.

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In the analysis of the soil and of organic fractions extracted from it the following analytical methods were used, viz.: organic matter was estimated by dichromate oxidation (9); nitrogen was determined by a microkjeldahl method (2); carbohydrate was estimated by use of anthrone (3); and uronic acid was estimated by a carbazole method (8).

Data on the composition of the organic fractions isolated are given in Table I, which records, first, the percentage of the organic matter of the soil sample found in each fraction, and then gives the percentages of nitrogen, carbohydrate and uronic acid estimated to be present in them.

TABLE 1.

| Fraction | Per cent of Soil Organic Matter | Percentages in Fractions | | |
|---|---|---|---|---|
| | | Nitrogen | Carbohydrate | Uronic Acid |
| A1 | 3.8 | 5.6 | 16.6 | 3.3 |
| A2 | 11.2 | 1.5 | 0.0 | 2.8 |
| A3 | 9.8 | 2.8 | 0.0 | 2.8 |
| A4 | 3.8 | 2.5 | 1.0 | 4.8 |
| B1 | 38.6 | 2.0 | 1.5 | 0.0 |
| B2 | 11.4 | 0.5 | 1.5 | 0.0 |

The method used for estimation of uronic acid gave positive results for fractions yielding no carbohydrate reaction with anthrone. The cause of this discrepancy was not established, but it is possible that polymerization subsequently found to occur on treatment with mineral acid inhibited the anthrone reaction, or that the estimates of uronic acid were too high because of reaction of carbazole with sulphydryl compounds (4).

The nitrogen content of the extracted organic matter, taken as a whole, was 2 per cent. Even if wholly present as protein such nitrogenous material would account for only about 13 per cent of the organic matter extracted. The apparent carbohydrate content of the organic matter removed from the soil amounted to but 1.8 per cent. Despite the uncertainty of this value, it seems highly probable that carbohydrate constituted but a small percentage of the organic matter extracted.

Following removal by a cation resin of any metallic ions remaining in the fractions precipitated by LPC (A2, A3 and A4), these were found to have neutralizing equivalents of 163, 152 and 145 respectively.

Application of the Forsyth technique for the separation and fractionation of 'fulvic acid' (6) to each of the organic fractions separated failed to reveal complexity of constitution. Thus, after adjustment of aqueous solutions of these fractions to pH 2.5, almost all of the organic matter of each was firmly adsorbed by charcoal, from which it was released only by use of 0.5N NaOH. However, on application of paper chromatography to aqueous solutions of these fractions, using aqueous n-propanol (n-propanol/water, 2 : 3, v/v) as solvent, it was found that fractions A1 and A3 each yielded one visible mobile product ($R_f$ values 0.66 and 0.74 respectively), while fractions A2 and A4 were not mobile in this solvent. Further, it was found that only the fractions from the benzene phase were stained by treatment with diazotized p-nitroaniline (PNA). The latter compound is expected to couple with phenols or aromatic amines having available a free position ortho or para to the coupling OH (or amine) group, to form brightly coloured azo dyes. This reaction is generally specific to phenols and aromatic amines (5).

Attempts were made to hydrolyze the organic fractions isolated using HCl, and also $H_2SO_4$, under reflux, each acid at concentrations ranging from 1.0 to 6.0 N and for periods ranging from 6 to 48 hours. In case of all fractions except A1 and A2, dark brown precipitates not appreciably soluble in water were formed on refluxing with these acids. After removal of mineral acid, examination of the hydrolyzates by paper chromatography failed to reveal the presence of either sugars or uronic acids in any of these solutions except that from fraction A1. From the latter, evidence for the presence of very small amounts of galacturonic acid, glucose, galactose, arabinose and xylose was obtained. Paper chromatograms from each of the hydrolyzates showed very faint evidence of the presence of ninhydrin-reactive substances, none of which was identified. Paper chromatography failed to reveal the presence of more than trace amounts of phenolic materials in these hydrolyzates. Such observations suggested the occurrence of polymerization on treatment of these organic fractions with hot mineral acids. Attempted hydrolysis with KOH solutions yielded similar results.

Attention was turned to fusion with KOH. The optimum fusion temperature was found to be between 250° and 300° C. Best control of the reaction was secured by fusion of the barium salts of the organic fractions with KOH, at a w/w proportion of about 1 : 45, in a nickel crucible over an open flame, beginning with very gentle heating, gradually increasing the temperature to the desired level, then continuing heating until frothing ceased and colour due to organic matter had just disappeared.

Fusion products extractable from an acidified aqueous solution of the melt were investigated. All the fractions isolated, except the small A1, were examined in this way. Four fractions of fusion products were obtained, viz.:

(a) Removed from acidified aqueous solution by ether and extracted from ether into aqueous $NaHCO_3$ solution;

(b) Volatile on steam distillation of the residue obtained by evaporation of ether after removal of (a);

(c) Not volatile on steam distillation of the residue obtained by evaporation of ether after removal of (a);

(d) Removed from acidified aqueous solution by ethyl acetate after extraction with ether.

The amounts of material recovered from the fusion melts of the five

TABLE II.

| Fraction | Ether-soluble | | | Ethyl Acetate Extract | Total |
|---|---|---|---|---|---|
| | Bicarbonate Extract | Steam Distillable | Not Steam Distillable | | |
| A2 | 36.2 | 7.5 | 10.7 | 10.9 | 65.3 |
| A3 | 31.5 | 17.5 | 17.5 | 4.1 | 70.4 |
| A4 | 38.0 | 8.4 | 6.2 | 11.3 | 63.9 |
| B1 | 17.3 | 7.3 | 7.5 | 9.8 | 37.7 |
| B2 | 16.5 | 17.5 | 10.8 | 11.8 | 54.0 |

organic fractions investigated, expressed as percentages of the organic matter fused, are given in Table II.

All the fusion products isolated in the above manner gave very strong strains when spotted on filter paper and treated with PNA or diazotized sulphanilic acid. This fact, coupled with the solubility relationships of these substances, suggested the presence of phenolic acids and/or phenols. Application of paper chromatography to aqueous solutions of these fusion products, using isopropanol-$NH_4OH$-$H_2O$ (8 : 1 : 1; v/v) as solvent, resulted in separation of nineteen phenolic compounds, thirteen of which were acidic. Fractions B1 and B2 each yielded the same nine phenolic substances, four of which were not obtained from any of the A fractions fused, while the remaining five also were present in all the A fractions examined. The three A fractions yielded a total of fifteen phenolic compounds, nine of which were common to all these fractions. The relative $R_f$ values of phenolic products numbered 11, 12 and 13 found in all five fractions fused, and of number 10 phenolic common to all three A fractions, and the staining reactions of these compounds, were found to be similar to those which other workers (1, 10) had reported for m-hydroxy, p-hydroxy-, 2,4-dihydroxy- and 3,5-dihydroxybenzoic acids. Fusion of 0.300 gm. of organic matter from fraction A3, known to yield fusion products 10 to 13 inclusive, followed by chromatographic separation of the fusion products on a column of cellulose powder using the 8 : 1 : 1 solvent, resulted in separation of sufficient amounts of these four substances to permit of determination of $R_f$ values, melting points, mixed melting points, and the absorption spectra of these compounds and of their coupling products with PNA. The values so obtained were compared with the corresponding values found for authentic compounds with which they were suspected to be identical; the data obtained are given in Table III.

TABLE III.

| Substance | $R_f$ Value | m.p. C° | Absorption Peaks (mμ) | |
|---|---|---|---|---|
| | | | Substance | PNA Coupling Product |
| No. 10 | 0.43 | 233—235 | 250, 305 | |
| 3,5—diOH | 0.43 | 234—235 | 250, 305 | 451 |
| Mixture | | 233—235 | | |
| No. 11 | 0.47 | 211—213 | 254 | |
| p—OH | 0.47 | 213 | 254 | 408, 507 |
| Mixture | | 211—213 | — | |
| No. 12 | 0.52 | 234—236 | 256, 295 | |
| 2,4—diOH | 0.52 | 235—236 | 2 | |
| Mixture | | 235—236 | | |
| No.13 | 0.61 | 199—202 | 295 | |
| m—OH | 0.61 | 202 | 295 | 416, 507 |
| Mixture | | 200—202 | | |

It was concluded that substances, 10, 11, 12 and 13 were 3,5-hydroxy-, p-hydroxy-, 2,4-dihydroxy- and m-hydroxybenzoic acids respectively.

For quantitative estimation, known amounts (5—15 μg) of each of these phenolic acids isolated from fusion melts were chromatogramed using the 8 : 1 : 1 solvent, stained with PNA, the coupled products eluted with 0.5

per cent ethanolic KOH and the optical densities of the solutions measured at 451, 507, 443 and 507 mu respectively. Estimations of the amounts present were based on standard curves obtained from authentic samples of these acids treated similarly. The results expressed as percentages of the material obtained by sodium bicarbonate extraction of ether extracts from acidified aqueous solutions of the fusion products of the five organic fractions investigated are given in Table IV.

Table IV.

| Fraction | m-Hydroxy | p-Hydroxy | 2,4-Dihydroxy | 3,5-Dihydroxy | Total |
|---|---|---|---|---|---|
| A2 | 27.3 | 35.7 | 13.4 | 10.1 | 86.5 |
| A3 | 21.1 | 35.0 | 10.5 | 9.3 | 75.9 |
| A4 | 9.5 | 11.9 | 4.7 | 2.4 | 28.5 |
| B1 | 28.7 | 28.1 | 9.6 | 0.0 | 66.4 |
| B2 | 33.6 | 34.2 | 10.4 | 0.0 | 78.2 |

The total amounts of these four phenolic acids found in the fusion products from the five organic fractions was equivalent to about 12 per cent of the total organic matter content of the soil. Some of the other fifteen phenolic compounds separated seemed to occur in small amounts only. However, the staining reactions of seven of these fifteen compounds suggested that they were present in quantities similar to those found for the two mono-hydroxybenzoic acids identified. The results indicate that substances obtained from extracts of the fusion melts of the organic fractions examined were mainly phenolic in nature. Further, since 75 to 80 per cent of the organic matter of the soil was extracted, and since the fusion products obtained amounted to from 38 to 70 per cent of the organic matter fused (Table II), the phenolic products obtained account for a considerable proportion of the organic matter of the soil studied.

Evidence has also been obtained in this laboratory (11) that leachates resulting from natural rainfall collected after passage through a canopy of hemock (*Tsuga canadensis*), and then through the O layer and the $A_2$ horizon of the gley podzol beneath, contained organic matter which, on fusion with KOH and analysis of the fusion products in the manner here described, yielded phenolic products, three of which were identified as p-hydroxy-, m-hydroxy- and 3,5-dihydroxy benzoic acids.

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## SUMMARY

The soil was obtained from the $B_{21}$ horizon of a well-drained sandy podzol under deciduous forest with *Fagus grandifolia* the major component. About seventy-five per cent of the organic matter of the soil was extracted by mechanical agitation of the system soil-water-benzene-8-quinolinol while maintaining the aqueous phase at pH 6.0 to 6.5.

Treatment of the organic matter of the aqueous phase with laryl-pyridinium chloride yielded four fractions; two fractions were obtained from the benzene phase.

Five of the fractions appeared to be dominantly aromatic. Following alkali fusion nineteen phenolic substances were separated chromatographically. The fusion products accounted for forty to seventy per cent of the organic matter of these fractions. Four of the products were identified as m-hydroxy-, p-hydroxy-, 2 : 4 dihydroxy- and 3 : 5 dihydroxy benzoic acids respectively. Quantitative colorimetry showed that these four compounds accounted for twelve per cent of the total organic matter content of the soil.

## RÉSUMÉ

L'échantillon étudié fut prélevé dans l'horizon $B_{21}$ d'un Podsol sableux bien drainé, sous forêt caducifoliée où *Fagus grandifolia* était l'espèce dominante. 70 % environ de la matière organique du sol fut extraite par agitation mécanique du système sol-eau-benzène-8-quinolinol, tout en maintenant le pH de la phase aqueuse entre 6.0 et 6.5.

En traitant ensuite la matière organique de la phase aqueuse par du chlorure de laryl-pyridine quatre fractions furent isolées. Deux autres fractions furent obtenues de la phase benzénique.

Cinq de ces fractions étaient surtout aromatiques. Après fusion alcaline on a pu séparer, par chromatographie, dix-neuf substances phénoliques. Les produits de fusion constituaient entre 40 et 70 % de la matière organique de ces fractions. Les quatre produits suivants ont pu être identifiés: acide m-hydroxy-benzoïque, acide p-hydroxy-benzoïque, acide 2 : 4 dihydroxy-benzoïque et acide 3 : 5 dihydroxy-benzoïque. La colorimétrie quantitative a montré que ces quatre produits constituaient 12 % de la matière organique totale du sol.

## ZUSAMMENFASSUNG

Die Bodenprobe wurde dem $B_{21}$-Horizont eines gut dränierten sandigen Podsols, mit sommergünem Waldbestanden worin *Fagus grandifolia* als Hauptkomponent, entnommen.

Ungefähr 75 % der organischen Substanz des Bodens liess sich extrahieren durch mechanisches Ausschütteln mit dem System Boden-Wasser-Benzen-8-Chinolin, dabei das pH der wässerigen Phase auf 6.0 bis 6.5 handhabend.

Behandlung der organischen Substanz in der wässerigen Phase mit Laryl-Pyridinchlorid lieferte 4 Fraktionen; die Benzenphase zwei Fraktionen.

Fünf der Fraktionen erwiesen sich hauptsächlich aromatisch zu sein. Nach nun folgender Alkalischmelze wurden neunzehn Phenolsubstanzen chromatographisch geschieden. Die Schmelzprc
bis siebzig % der organischen Substanz dieser I
Produkte wurden resp. als m-Hydroxy-, p-Hydroxy-, 2 : 4 Dihydroxy- und 3 : 5 Dihydroxybenzoesäure identifiziert. Quantitative Kolorimetrie zeigte dass diese 4 Komponenten 12 % des totalen Gehaltes an organischer Substanz des Bodens ausmachten.

## DISCUSSION

W. FLAIG: M-hydroxy-, and 3,5-dihydroxy-benzoic acids are normally not decomposition products of lignins. Is it possible that these acids are formed by the decomposition of tannins?

D. E. COFFIN: It is possible that these acids could be formed by the decomposition of tannins or similar substances. However, I doubt if tannins would fit some of the other results reported in this paper.

CORNELIUS STEELINK: Does your extraction correspond to a typical benzene-alcohol extraction for soluble substances, such as fats, waxes, resins, etc.?

D. E. COFFIN: We also extracted the same soil with a similar system except that 8-quinolinol was omitted. An amount of soil which had yielded 4 to 5 grams of organic material when 8-quinolinol was present in the extracting system, yielded approximately 40 milligrams of organic material when 8-quinolinol was omitted. Thus, it appears that benzene-soluble materials such as fats, waxes, and resins did not constitute any appreciable portion of the organic material in this soil.

L. ALEXANDROVA: Are these phenolic substances the individual compounds or are they in the macromolecule of fulvic acids?

D. E. COFFIN: These phenolic acids are definitely degradative products of the macromolecule. They do not exist as individual compounds in the soil.

CORNELIUS STEELINK: Did you find any typical lignin fusion products, such as vanillic acid, protocatechuic acid, and pyrocatechol? Your phenolic compounds apparently are not lignin-derived.

D. E. COFFIN: We were not able to identify any of these products. They are the products which we originally expected to obtain, but none of these were present in our preparations.

H. DEUEL: Have you isolated polysaccharides from your fraction $A_1$?

D. E. COFFIN: No. Since we were interested primarily in the nature of the major portion of the organic material, no attempt was made to isolate polysaccharides.

R. BERNIER: In your first slide, you reported 4.8 % uronic acid and yet very little sugar. Are these uronic acids part of a polysaccharide? If so, I don't see why you do not find also the presence of sugars in the hydrolysates.

D. E. COFFIN: In this instance we are working with two methods of analysis and we are not sure that either is specific. We think that the uronic acid value is in error, as we have been unable to detect any uronic acids in the hydrolysates of this particular fraction.